

Fibroblast Invasive Migration into Fibronectin/Fibrin Gels Requires a Previously Uncharacterized Dermatan Sulfate-CD44 Proteoglycan

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After tissue injury, fibroblast migration from the peri-wound collagenous stroma into the fibrin-laden wound is critical for granulation tissue formation and subsequent healing. Recently we found that fibroblast transmigration from a collagen matrix into a fibrin matrix required the presence of fibronectin. Several integrins— $\alpha 4\beta 1$, $\alpha 5\beta 1$, and $\alpha v\beta 3$ —with known fibronectin binding affinity were necessary for this invasive migration. Here we examined another family of cell surface receptors: the proteoglycans. We found that dermatan sulfate was required for fibroblast migration into a fibronectin/fibrin gel. This conclusion was based on β -xyloside inhibition of glycanation and specific glycosaminoglycan degradation. CD44, a cell surface receptor known to bind hyaluronan, not infrequently exists as a proteoglycan, decorated with various glycosaminoglycan chains including heparan sulfate and chondroitin sulfate, and as such can bind fibronectin. We found that CD44H, the non-spliced isoform of CD44, was necessary for fibroblast invasion into fibronectin/fibrin gels. Resting fibroblasts expressed mostly nonglycanated CD44H core protein, which became glycanated with chondroitin sulfate and dermatan sulfate, but not heparan sulfate, after a 24 h incubation with platelet-derived growth factor, the stimulus used in the migration assay. These results demonstrate that dermatan sulfate-CD44H proteoglycan is essential for fibroblast migration into fibrin clots and that platelet-derived growth factor, the stimulus for migration, induces the production of chondroitin-sulfate- and dermatan-sulfate-glycanated CD44H.

Key words: CD-44/cell migration/chondroitin sulfate/collagen/fibroblast/glycosaminoglycan/heparan sulfate/wounds.

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Fibroblasts are normally quiescent mesenchymal cells, which are surrounded by collagenous extracellular matrix (ECM). After tissue injury, however, fibroblasts in proximity to the wound become activated (McClain *et al*, 1996). During the first 3 d postinjury, fibroblasts proliferate in the peri-wound stroma. On the fourth day, fibroblasts transmigrate from the collagen-rich connective tissue into the wound provisional matrix, composed mainly of fibrin and fibronectin (FN) (Clark, 1996). In this provisional matrix, fibrin forms a three-dimensional scaffold whereas FN provides a conduit for fibroblast migration (Greiling and Clark, 1997).

The necessity of FN for fibroblast movement across a collagen–fibrin matrix boundary was recently demonstrated using an *in vitro* model of cell movement in a discontinuous three-dimensional tissue construct that simulates early wound repair (Greiling and Clark, 1997). FN had to be present in both the collagen gel and the fibrin gel for migration to proceed. Furthermore, the integrins $\alpha v\beta 3$ and

$\alpha 5\beta 1$ were also required for this cell transmigration. Integrins comprise a superfamily of heterodimeric, transmembrane cell surface receptors, many of which mediate cell adhesion to ECM proteins (Hynes, 1992). When ligated, these receptors transmit intercellular signals (Clark and Brugge, 1995) and physically connect to the cytoskeleton (Gumbiner, 1996). It is not surprising that they have been implicated in cell movement (Huttenlocher *et al*, 1996). Nevertheless, other cell surface receptors, including transmembrane prostaglandins (PG), may be involved in cell motility (McCarthy *et al*, 1996).

Structurally, PG consist of a core protein to which linear glycosaminoglycans (GAG), such as heparan sulfate (HS) or chondroitin sulfate (CS), are attached via an O-glycosidic linkage. PG can modulate adhesion by binding ECM proteins or receptors on other cells (Milstone *et al*, 1994; Jackson *et al*, 1995; Woods *et al*, 1998). Furthermore, cell surface PG can collaborate with integrins in activating intracellular signaling pathways. For example, fibroblasts plated on FN required both cell surface PG and integrins to spread fully, forming focal contacts and stress fibers (Woods *et al*, 2000). Interestingly, PG binding sites are often expressed in close proximity to integrin binding domains within ECM molecules, suggesting that cellular recognition

Abbreviations: CD44H, standard or hematopoietic form of CD44; CS, chondroitin sulfate; DS, dermatan sulfate; FN, fibronectin; GAG, glycosaminoglycan; HA, hyaluronan; HS, heparan sulfate; MESF, molecules of equivalent soluble fluorochrome.

of the ECM might involve the formation of clusters including both cell surface PG and integrins (Iida *et al*, 1996).

CD44 is a widely expressed cell surface adhesion receptor, which can be glycanated with either CS or HS (McCarthy *et al*, 1996). CD44 represents a family of molecules, all encoded by one gene. Isoform variability is generated by post-transcriptional RNA splicing. Up to 10 alternative exons (v1–v10) may be inserted into the mRNA (Brown *et al*, 1991; Dougherty *et al*, 1991; He *et al*, 1992; Jackson *et al*, 1992). In addition, the isoforms can undergo extensive post-translational modifications including N- and O-linked glycosylation and O-linked glycanation with GAG such as HS and CS (Brown *et al*, 1991; Faassen *et al*, 1992; 1993; Greenfield *et al*, 1999). The “standard” or “hematopoietic” nonglycanated form of CD44 (CD44H), found on most cell types, has an apparent size of 85k to 95k on gel electrophoresis, but its amino acid sequence predicts a polypeptide of less than 40k molecular weight (Borland *et al*, 1998). This difference results from extensive glycosylation of its extracellular domain. CD44H lacks all 10 alternative exons but can be glycanated with CS at the first serine-glycine site in exon 5 of CD44 (Greenfield *et al*, 1999). Alternatively spliced isoforms of CD44 are largely restricted to cells of epithelial origin, but can also occur in cultured lymphocytes and monocytes spontaneously (Levesque and Haynes, 1996) or after treatment with mitogenic agents or cytokines (Koopman *et al*, 1993; Mackay *et al*, 1994) and in cancer cells (Lesley *et al*, 1997; Goodison and Tarin, 1998). CD44 is the principal cell surface receptor for hyaluronan (HA) (Underhill, 1992); however, not all CD44-expressing cells bind HA. Both glycosylation and glycanation can affect HA binding (Borland *et al*, 1998). When glycanated with CS, CD44 can bind collagen and FN (Culty *et al*, 1990; Jalkanen and Jalkanen, 1992).

Several facts suggest that CD44 may play a role in fibroblast migration in wound healing. CD44 can be a receptor for FN or HA, components of the provisional matrix that facilitate cell migration into a wound site (Knox *et al*, 1986; McCarthy *et al*, 1986; Turley, 1992; Clark, 1996; Greiling and Clark, 1997). Increased CD44 expression occurs in hypertrophic scar fibroblasts compared to normal dermal fibroblasts (Messadi and Bertolami, 1993). Finally, Svee *et al* (1996) showed that human lung fibroblast invasion into a fibrin gel required a nonglycanated CD44H. In this study we demonstrated that human dermal fibroblast transmigration from collagen matrices into fibrin clots requires cell surface heparan and dermatan sulfate (DS) proteoglycans. The latter was attributable to a DS-CD44H that has not been previously characterized. Resting dermal fibroblasts expressed mostly nonglycanated CD44H. After a 24 h incubation with platelet-derived growth factor (PDGF), however, a well-known growth factor and chemotactic factor for fibroblasts *in vitro* and an important factor for wound healing *in vivo* (Heldin and Westermark, 1996), CD44H became glycanated with DS and CS.

Results

Invasive migration of human dermal fibroblasts requires GAG

The aim of this study was to investigate if proteogly-

can receptors are functionally involved in the transmigration of normal adult human dermal fibroblasts from a collagen matrix into a fibrin gel. Therefore we investigated the requirement of cell surface CS/DS as it has been shown by several other groups that CS side chains are critical for adherence of some cells to FN (Faassen *et al*, 1992; Jalkanen and Jalkanen, 1992). For this purpose, β -xyloside was added to the fibrin gel, which, at the chosen concentration of 1 mM, prevents the addition of CS/DS GAG chains to proteoglycan core proteins (Schwarz, 1977). As a negative control, α -xyloside, an inactive homolog of β -xyloside, was added to the assay. β -Xyloside inhibited transmigration by about 90% (Fig 1A). The treatment with α -xyloside did not have any effect on the invasion of fibroblasts into the fibrin gel. A longer exposure of the cells to this substance by pretreatment of the contracted collagen gels for 24 h did not change the results (data not shown).

Invasive migration of dermal fibroblasts requires DS To confirm the involvement of CS or DS in cell migration, cells embedded in collagen gel were exposed to chondroitinase ABC, an enzyme that degrades all forms of CS from the cell

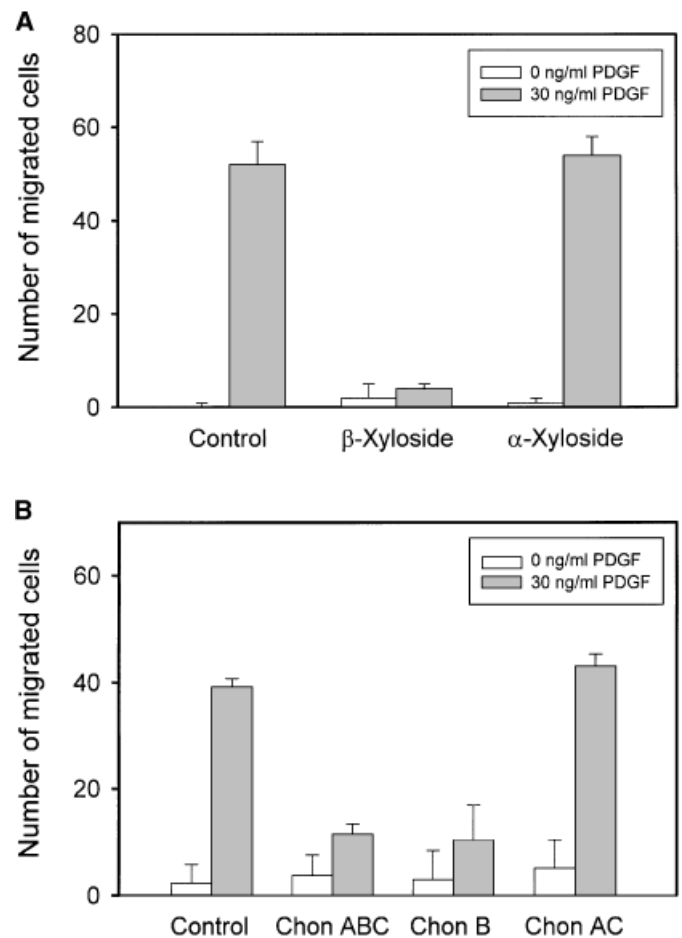


Figure 1
Fibroblast transmigration requires a DS proteoglycan. (A) β -Xyloside decreased PDGF-induced transmigration from a collagen matrix into a fibrin gel. The inactive analog α -xyloside did not change the number of migrated cells. (B) Chondroitinase ABC and chondroitinase B inhibited PDGF-induced transmigration. Chondroitinase ACII failed to influence the amount of accumulated cells in the fibrin gel. The data are shown as mean \pm SEM and are representative for three independent experiments.

surface: chondroitin-4-sulfate and chondroitin-6-sulfate, as well as DS (Aruffo *et al*, 1990). The enzyme was also present during the assay. Transmigration was inhibited by 65% at 0.1 U per mL (Fig 1B). To determine which specific galactosaminoglycan was required for transmigration, chondroitinases ACII and B were added to the assay. Chondroitinase ACII digests chondroitin-4-sulfate and chondroitin-6-sulfate, whereas chondroitinase B is specific for DS. Like chondroitinase ABC chondroitinase B decreased the number of transmigrated cells whereas chondroitinase AC did not influence the migration at all (Fig 1B). Both enzymes were used at 0.1 U per mL. From these data we conclude that transmigration of fibroblasts from a collagen gel into a fibrin gel requires a cell surface galactosaminoglycan, specifically DS.

The lack of migration in the presence of chondroitinase was not due to contaminating protease activity as addition of ovomucoid (10 μ g per mL), a broad-spectrum protease inhibitor, did not change the results (data not shown). None of the substances used to alter GAG expression, including β -xyloside, α -xyloside, chondroitinase ABC, chondroitinase AC, and chondroitinase B, had a toxic effect on the fibroblasts as judged by the LDH assay (Sigma, St Louis, MO). The assay was done as suggested by the supplier. Greater than 95% LDH was retained in fibroblasts after a 24 h incubation at 37°C with each test substance.

Invasive migration of dermal fibroblasts requires CD44 CD44 can be glycanated with galactosaminoglycans and/or glucosaminoglycans. Therefore, a CD44 proteoglycan might be required for adult dermal fibroblast migration through fibrin gels. This possibility was examined by the addition of antibodies against different CD44 epitopes to the fibroblast transmigration assay. In Fig 2(A) antibodies were added to the fibrin gel at a final concentration of 30 μ g per mL. Only monoclonal antibody BU52 did not influence transmigration compared to control (Co). Monoclonal antibody A1G3 enhanced cell movement, whereas BRIC 214, BRIC 235, Hermes-3, and A3D8 inhibited it. To further study the stimulatory and inhibitory antibodies, A1G3 and A3D8, respectively, their effect on migration was observed in the concentration range 1–100 μ g per mL. As shown in Fig 2(B), monoclonal antibody A3D8 inhibited the transmigration in a dose-dependent manner with an IC_{50} at about 4 μ g per mL, whereas monoclonal antibody A1G3 increased the invasive migration of fibroblasts in a dose-dependent manner with the optimal effect at 30 μ g per mL.

A previous report demonstrated that CD44 antibodies induced fibroblast apoptosis (Henke *et al*, 1996a). Using a sensitive assay for DNA fragmentation (Sugano *et al*, 1999), only slight apoptosis was observed with monoclonal antibody A3D8 (Fig 3), a monoclonal anti-CD44 antibody when used at concentrations of 10 μ g per mL and higher completely inhibited fibroblast migration through fibrin gels (Fig 2B). An equal amount of apoptosis was detected with monoclonal antibody A1G3 (Fig 3), a monoclonal anti-CD44 antibody when used at concentrations of 10 μ g per mL and higher greatly increased fibroblast migration through fibrin gels (Fig 2B). Thus monoclonal antibody A3D8 inhibition of migration could not be attributed to fibroblast apoptosis.

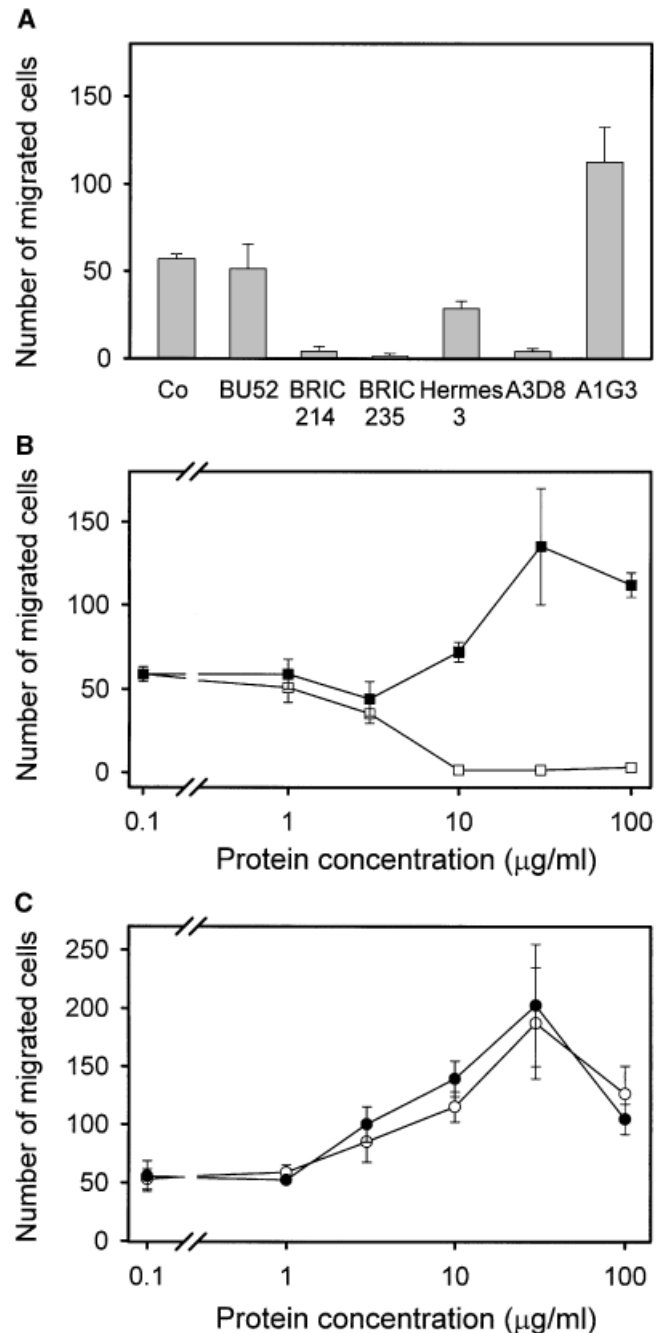
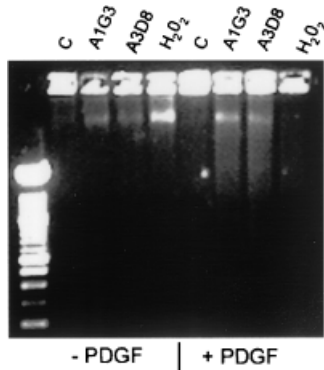


Figure 2

Fibroblast transmigration requires CD44. (A) Antibodies directed against different epitopes of CD44 have a differential effect on fibroblast transmigration. The control (Co) had no antibody addition. (B) Monoclonal antibody A3D8 (open squares) inhibited PDGF-induced transmigration whereas A1G3 (closed squares) enhanced the number of migrated cells. (C) Enhancement of PDGF-induced transmigration by monoclonal antibody A1G3 (open circles) was not influenced by the presence of hyaluronidase (closed circles). Data are shown as mean \pm SEM and are representative of two independent experiments.

A1G3 has been reported to induce HA binding of the epithelial form of CD44 (Liao *et al*, 1993), a CD44 isoform that normally does not have this capability (Stamenkovic *et al*, 1989; 1991). To exclude a role of HA on the enhanced migration observed with A1G3 in our assay system, *Streptomyces* hyaluronidase (2 U per mL) was added to the fibrin gel. Such treatment had no effect on the dose-

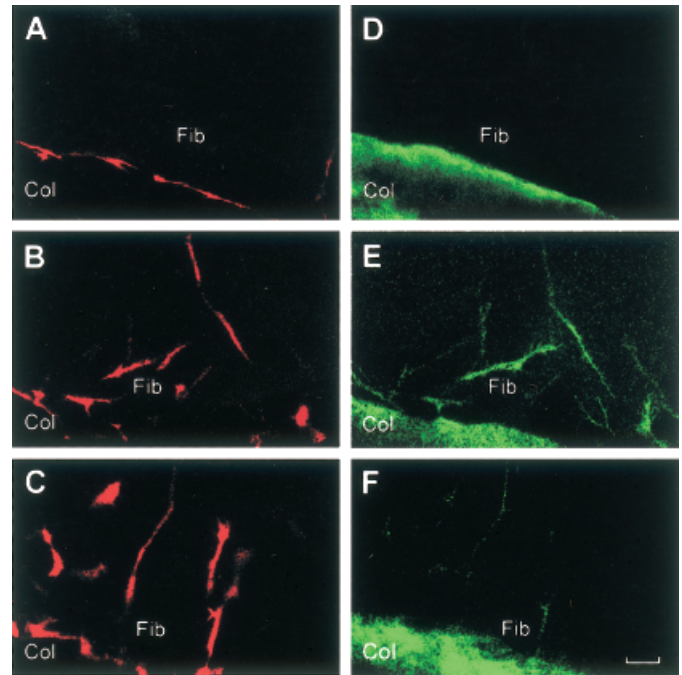
**Figure 3**

DNA fragmentation in fibrin gel. Fibroblasts were incubated with or without anti-CDE44 antibody (10 μ g per mL) or H_2O_2 (5 μ M) in the presence or absence of PDGF in fibrin gel for 24 h. DNA was extracted and analyzed by electrophoresis on a 1.5% gel. DNA was visualized with ethidium bromide using a Chemilmager 440 imaging system (Imgen Technologies). The results shown are representative of three separate experiments performed on separate occasions. C, no treatment control.

response curve of A1G3 (Fig 2C). To confirm that hyaluronidase degraded HA deposited in the fibrin gel by migrating fibroblasts, HA was stained using a biotin-labeled HA binding fragment (Hendin *et al*, 1990). For this immunofluorescence experiment cells were visualized with propidium iodide (Fig 4, panels A, B, C). No HA was present in fibrin gels if no fibroblasts had migrated (Fig 4D). HA was present, however, in gels through which fibroblasts had migrated (Fig 4E). Hyaluronidase cleared gels of HA deposited by migrating fibroblasts (Fig 4F) but did not affect migration (Figs 2C, 4C).

Human dermal fibroblasts express only CD44H As a first step toward elucidating which CD44 isoform(s) human fibroblasts expressed, cells were labeled with antibodies against four different spliced variants of CD44 (CD44H, CD44v3, CD44v4/5, and CD44v6) and analyzed by quantitative flow cytometry. Initially fibroblasts and human keratinocytes as control cells, which are known to express CD44 spliced variants (Milstone *et al*, 1994), were cultured to 80% confluence on tissue culture plastic. As shown in Fig 5, the CD44H isoform (panel A), but not the spliced variants (panels B–D), was expressed by fibroblasts. In contrast, keratinocytes did not express the CD44H isoform (panel E), but rather expressed the spliced variants CD44v3 (panel F) and CD44v6 (panel H). PDGF and three-dimensional collagen matrix can have an enormous effect on ECM receptor expression (Xu and Clark, 1996). Therefore, fibroblasts were cultured either on plastic or in collagen gels in the absence or presence of 30 ng per mL PDGF for 48 h (Table I). Neither PDGF nor collagen matrix induced fibroblast expression of CD44 variant forms.

To confirm that human adult dermal fibroblasts expressed only CD44H, we determined the relative molecular weight of all CD44 moieties present in fibroblasts in the presence or absence of 30 ng per mL PDGF for 48 h. Western blots of fibroblast extracts were probed with the monoclonal antibody BBA10, which recognizes all CD44 isoforms (Fig 6). Some fibroblast monolayers were treated

**Figure 4**

Confocal microscope images of fibrin gel migration assays double-labeled for cells with propidium iodide (A–C) and HA with HA-binding peptide (D–F). Fibrin gels (Fib) in the absence of PDGF demonstrated no fibroblast migration (A) and no HA (D). Collagen gels (Col) are autofluorescing under the filter settings for FITC (D–F). Fibroblasts are present in fibrin gels (Fib) incubated with PDGF for 24 h (B). Migrating fibroblasts and the surrounding fibrin gel (Fib) stain positive for HA (E). (C), (F) PDGF-stimulated cell migration assay in which fibrin gels were treated with 10 U per mL hyaluronidase. Migrating cells (C) are not associated with green fluorescence (F). Bar: 10 μ m.

with 0.2 U per mL chondroitinase ABC prior to extraction whereas control monolayers were treated with buffer alone. Cells cultured in the absence of PDGF gave a prominent CD44 band located at 85k (Fig 6, lane 1) consistent with CD44H. After incubation with chondroitinase ABC, CD44 from such non-stimulated cells also ran at 85k (Fig 6, lane 2). CD44 from cells incubated with 30 μ g per mL PDGF gave a large smear at and above 85k (Fig 6, lane 3); CD44 from such cells treated with chondroitinase ABC located in a discrete band at 85k (Fig 6, lane 4). Thus, human adult dermal fibroblasts, with or without PDGF stimulation, expressed only an 85k CD44 core protein, a relative molecular weight consistent with CD44H.

PDGF induced dermal fibroblasts to express CS-CD44H and DS-CD44H

To determine whether fibroblast CD44H was glycanated with CS and/or DS, western blots of extracts from fibroblasts with or without PDGF stimulation were probed with a mixture of anti- Δ Di-OS, Δ Di-4S, and Δ Di-6S monoclonal antibodies against chondroitin “stubs” (Fig 7). Without chondroitinase treatment little or no chondroitin stubs were observed in non-stimulated cells (Fig 7, lane 1) and some chondroitin stub was observed in PDGF-stimulated fibroblasts (Fig 7, lane 5). The latter result may be secondary to incomplete GAG synthesis of CD44H as it transits through the endoplasmic reticulum. After treatment with chondroitinase ABC, a faint band at 85k was

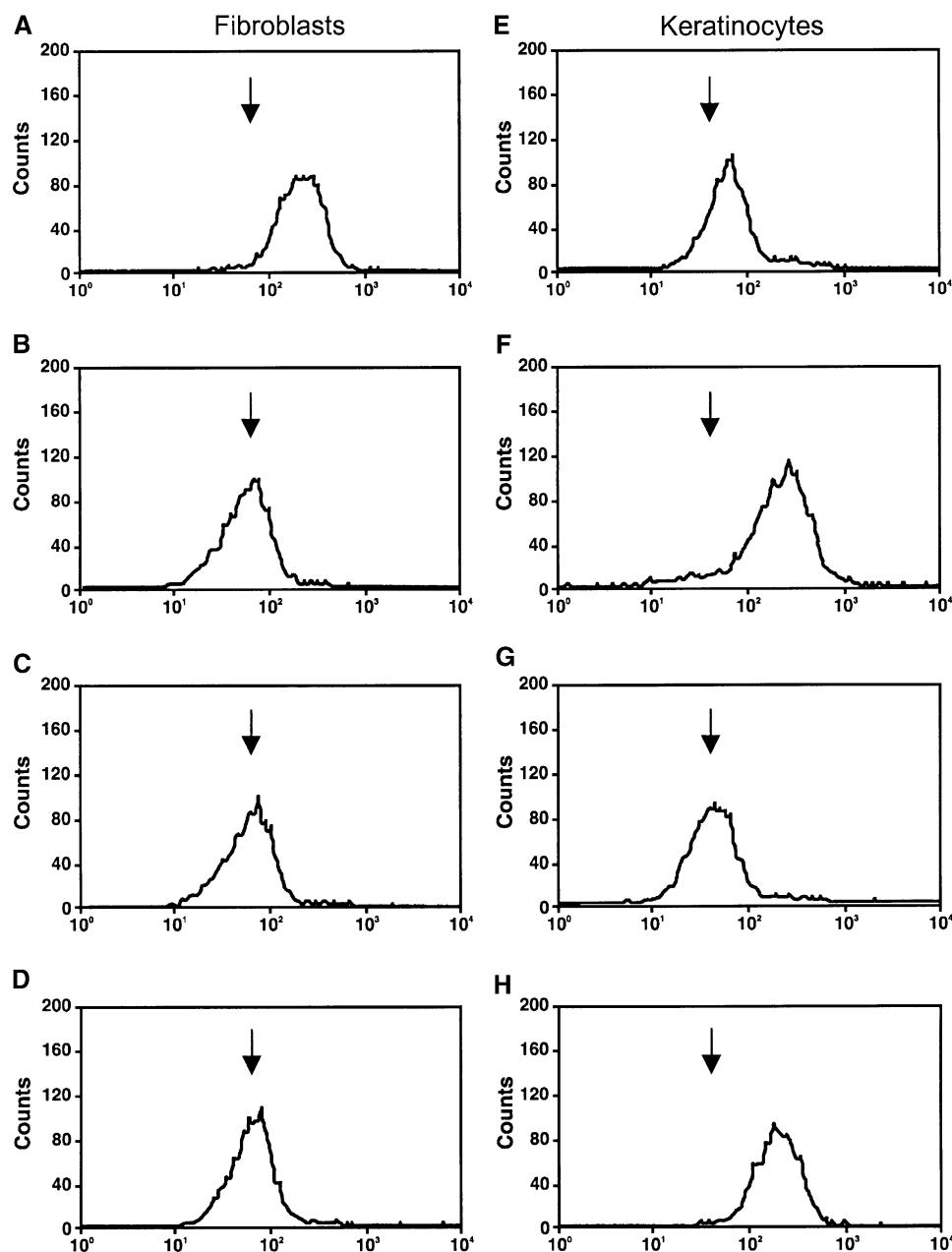


Figure 5
Adult human dermal fibroblasts only express CD44 isoform CD44H. Fluorescence-activated cell sorter analysis showed that fibroblasts expressed CD44H (A) whereas CD44v3 (B), CD44v4/5 (C), and CD44v6 (D) expression was not detectable as the pattern was the same as second antibody alone (arrows represent the maximum level of cell fluorescence in the absence of primary antibody). Human keratinocytes were negative for CD44H (E) but expressed CD44v3 (F) and CD44v6 (H).

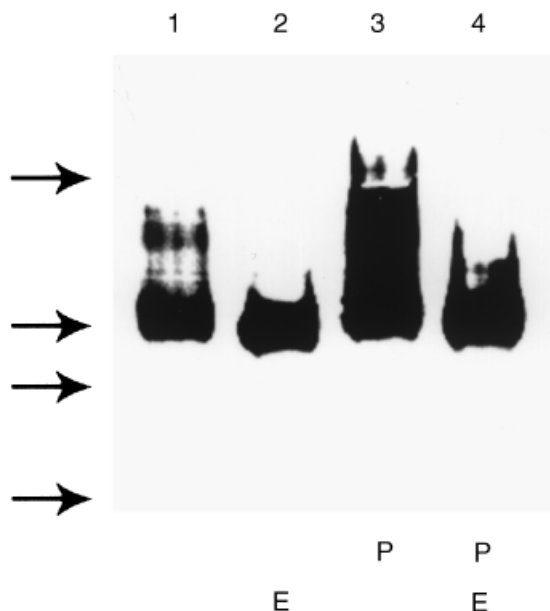
Table I. CD44 isoform expression on adult human dermal fibroblasts, determined by fluorescence-activated cell sorter analysis

CD44 isoforms	Fibroblasts in monolayer culture ^a		Fibroblasts embedded in collagen gel	
	0 ng per mL PDGF	30 ng per mL PDGF	0 ng per mL PDGF	30 ng per mL PDGF
CD44H	79,400 MESF	75,900 MESF	45,350 MESF	42,230 MESF
CD44v3	Undetectable	Undetectable	Undetectable	Undetectable
CD44v4/5	Undetectable	Undetectable	Undetectable	Undetectable
CD44v6	Undetectable	Undetectable	Undetectable	Undetectable

^aFibroblasts were cultured on tissue culture plastic or in collagen gels in the absence or presence of 30 ng per mL PDGF-BB for 48 h.

observed from extracts of non-stimulated cells (Fig 7, lane 2). Little or no signal was detected in non-stimulated cell extracts after treatment with chondroitinase AC or B (Fig 7, lanes 3 and 4, respectively). In contrast, fibroblasts stimulated with PDGF showed abundant chondroitin stub

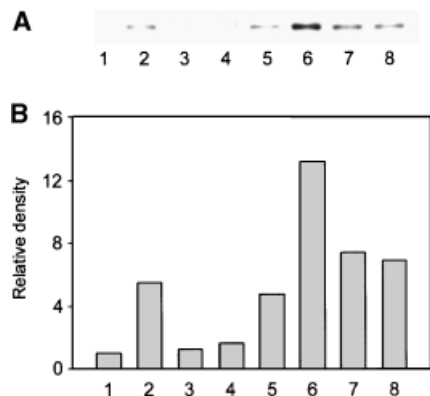
after treatment with chondroitinase ABC (Fig 7, lane 6) and modest chondroitin stubs after digestion with chondroitinase AC and chondroitinase B (Fig 7, lanes 7 and 8, respectively). Similar results were obtained when western blots of fibroblast extracts were probed with R36 polyclonal

**Figure 6**

Adult dermal fibroblasts express only 85k CD44 core protein regardless of stimulation with PDGF. The western blot was probed with monoclonal antibody BBA10 that recognizes all CD44 splice variants. Some cell monolayers were incubated with PDGF for 48 h prior to extraction (lanes 3, 4). Some cell monolayers were digested with chondroitinase ABC immediately before extraction (lanes 2, 4). Molecular weight markers (205 kDa, 105 kDa, 70 kDa, and 43 kDa) from the original SDS-PAGE are indicated on the left-hand side of the figure.

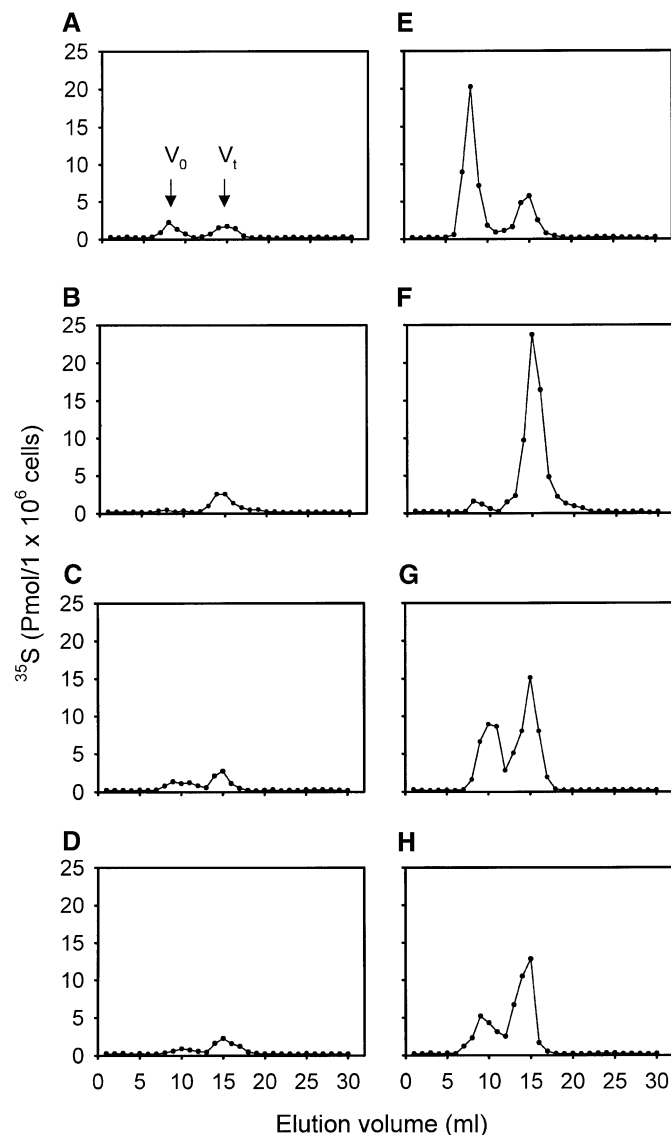
antibodies against the chondroitin stubs (data not shown). These data demonstrate that resting human dermal fibroblasts express mostly nonglycanated CD44H, which become glycanated with CS and DS chains after incubation with PDGF.

To confirm that PDGF induced human dermal fibroblasts to glycanate CD44H with sulfated GAG, cells were metabolically labeled with $^{35}\text{SO}_4$ in the absence or presence of PDGF (100 ng per mL) for 48 h. After incubation the cells

**Figure 7**

CD44H becomes glycanated with CS and DS after fibroblast exposure to PDGF. Western blots of fibroblast extracts were probed for CS/DS stubs with a mixture of anti- $\Delta\text{Di-OS}$, $\Delta\text{Di-4S}$, and $\Delta\text{Di-6S}$ monoclonal antibodies. Human adult dermal fibroblasts were incubated in the absence (lanes 1–4) or presence (lanes 5–8) of 30 ng per mL PDGF for 48 h. Immediately prior to extraction cells were incubated with no enzyme (lanes 1, 5), chondroitinase ABC (lanes 2, 6), chondroitinase AC (lanes 3, 7), or chondroitinase B (lanes 4, 8). All bands ran at 85k on SDS-PAGE.

were lysed and CD44 affinity purified on anti-CD44H/protein G/agarose beads, aliquots of which were differentially digested with chondroitinase ABC, AC, or B. After hydrolysis with $\text{NaOH}/\text{NaBH}_4$, reaction mixtures were passed through a G-50 Sephadex column and fractions were collected and counted. When experiments were done in 1% serum little CD44 labeling activity was detected in the presence or absence of PDGF (data not shown). When experiments were performed in the presence of 10% serum, however, the addition of PDGF substantially increased CD44 glycanation (Fig 8). In the absence of PDGF

**Figure 8**

Column chromatography of metabolically labeled, affinity-purified, differentially digested CD44 from human adult dermal fibroblasts demonstrates that CD44H becomes glycanated with galactosaminoglycans after PDGF stimulation. Cells were metabolically labeled with $^{35}\text{SO}_4$ in the absence (panels A–D) or presence (panels E–H) of PDGF (100 ng per mL) for 48 h. After the incubation period, affinity-purified CD44 was incubated with no enzyme (panels A, E), chondroitinase ABC (panels B, F), chondroitinase AC (panels C, G), or chondroitinase B (panels D, H). $\text{NaOH}/\text{NaBH}_4$ hydrolysates of the differentially digested CD44 moieties were passed through a G-50 Sephadex column and fractions were collected and counted. V_0 and V_t represent column exclusion volume and total volume, respectively.

little sulfated GAG was detected in fibroblast monolayers that had not been treated with enzyme (Fig 8A). Sulfated GAG were excluded from the G-50 Sephadex column (void volume 7 mL) whereas sulfated monosaccharides were retained in the column with a peak at 15 mL, the column total volume (V_t). No sulfated GAG was detectable after chondroitinase ABC treatment (Fig 8B) and little was detected when monolayers had been incubated with chondroitinase AC (Fig 8C) or chondroitinase B (Fig 8D). Fibroblasts that had been stimulated with PDGF for 48 h demonstrated a substantial increase in sulfated GAG synthesis (Fig 8E–H). Digestion of stimulated cells with either chondroitinase AC (Fig 8G) or chondroitinase B (Fig 8H) demonstrated the presence of DS and CS, respectively. The amount of DS observed after digestion with chondroitinase AC (first peak in Fig 8G) appeared to be about twice the amount of residual CS seen after chondroitinase B digestion (first peak in Fig 8H).

Discussion

After substantial tissue injury, the transmigration of fibroblasts and blood vessels from peri-wound collagenous stroma into the wound provisional matrix is the initial step of granulation tissue formation (McClain *et al*, 1996). The global aim of our laboratory is to understand the functional mechanisms involved in this complex process. The data presented here demonstrate that PDGF-induced transmigration of human adult dermal fibroblasts from a type 1 collagen matrix into an FN/fibrin provisional matrix is dependent on a previously uncharacterized CD44 proteoglycan, DS-CD44H.

Monoclonal antibody experiments demonstrated that CD44 was required for fibroblast invasive migration into FN/fibrin gels. The question arose, however, whether this was secondary to a direct interaction with FN or an interaction with HA deposited by fibroblasts as they migrated. Both BRIC 214 and BRIC 235 completely inhibited fibroblast invasive migration into FN/fibrin gels. Importantly, BRIC 235 had been shown to block interactions with HA (Liao *et al*, 1995; Bartolazzi *et al*, 1996). Furthermore, A1G3, which remarkably enhanced fibroblast transmigration in our studies, had been shown to enhance HA binding to CD44H and the epithelial form of CD44, a CD44 isoform that does not normally bind HA (Liao *et al*, 1993, 1995).

Thus, some monoclonal antibody data lead to the consideration that CD44 might be affecting migration indirectly through HA. The increased migration stimulated by A1G3, however, was not due to CD44 interaction with HA as removal of fibroblast-secreted HA with hyaluronidase did not change the antibody effect on migration. That the fibroblast migration observed here was HA independent is further supported by the fact that monoclonal antibody A3D8, which also enhances HA binding to CD44H (Liao *et al*, 1993; 1995), completely inhibited fibroblast invasive migration into FN/fibrin gels, rather than stimulating it. The ability of A3D8 to inhibit migration was not secondary to apoptosis or other cytotoxic effects. In fact, the small amount of DNA fragmentation and LDH release observed

was the same whether fibroblasts were exposed to A3D8 or A1G3. A previous study reported that A3D8 could induce apoptosis of fibroblasts cultured in fibrin gels (Henke *et al*, 1996a); however, our assay included PDGF, which is known to protect cells against apoptosis (Romashkova and Makarov, 1999).

CD44 may be modified with either CS or HS, or both, depending on the isoform being glycanated. Only isoforms with v3 can be substituted with HS (Bennett *et al*, 1995), presumably because only the v3 insert has a Ser-Gly-Ser-Gly consensus sequence, the preferential attachment site for HS. All other GAG attachment sites in CD44 consist of Ser-Gly residues in an environment preferential for galactosaminoglycan substitution. The expression of CD44 isoforms can change in response to differentiation and/or activation (Arch *et al*, 1992; Koopman *et al*, 1993). Therefore, human adult dermal fibroblasts were cultured in the absence and presence of PDGF for 48 h and subsequently analyzed for expression of CD44H, CD44v3, CD44v4/5, and CD44v6. In both conditions the fibroblasts expressed only CD44H. Keratinocytes, which are known to express CD44 transcripts that include v3 and v6 (Bennett *et al*, 1995; Bloor *et al*, 2001), were used as a positive control. As ECM has an enormous effect on PDGF induction of fibroblast integrin receptors (Xu and Clark, 1996), fibroblasts were also embedded in collagen gels to examine their effect on CD44 isoform expression. Again only CD44H was observed, even after incubation with PDGF for 48 h.

Treatment of fibroblasts with chondroitinase enzymes, chondroitinase ABC, chondroitinase ACII, and chondroitinase B, revealed that cell invasion of FN/fibrin was dependent on DS but not CS. Chondroitinase ABC degrades all forms of CS and DS and inhibited migration, whereas chondroitinase ACII cannot degrade iduronate-containing DS and did not inhibit migration. Conversely, chondroitinase B degrades DS but not CS chains and did inhibit migration. Previously, an uncharacterized DS proteoglycan was shown to be important for fibroblast proliferation (Denholm *et al*, 2000). In our 24 h assay, however, migration is independent of proliferation as it is unaffected by irradiation (Greiling and Clark, 1997).

Previously, Jalkanen and Jalkanen (1992) demonstrated that CD44H can be substituted with CS chains. In 1996 Ehnis *et al* demonstrated that a CS/DS form of CD44 is a receptor for collagen XIV. The evidence for the latter came from abrogation of binding with chondroitinase ABC, which digests both CS and DS. The CD44 proteoglycan responsible for collagen XIV binding was not further characterized.

To determine whether the CD44H on fibroblasts was glycanated, and, if so, to identify the type of GAG, we utilized the differential substrate specificity of chondroitinase enzymes, together with monoclonal and polyclonal antibodies, which recognize “stubs” remaining on the core protein after enzyme treatment. In the absence of PDGF, human dermal fibroblasts substituted a small amount of CD44H with galactosaminoglycans, but GAG levels were too low to discern whether they were CS, DS, or both. PDGF-treated dermal fibroblasts expressed CD44H substituted with greatly increased levels of both DS and CS chains. Although theoretically PDGF could induce CS or DS glycanation of other proteoglycan core proteins, only one

protein band was observed on western blot with "stub" antibodies after chondroitinase treatment. Furthermore, the protein recognized by "stub" antibodies ran at an apparent molecular weight of 85k consistent with CD44H (Borland *et al*, 1998). These data taken together indicate that overall glycanation of CD44H was upregulated by PDGF and suggest that a previously uncharacterized DS-CD44H was expressed after PDGF stimulation and necessary for human dermal fibroblast invasion of FN/fibrin. To confirm that CD44 was the core protein to which DS and CS were glycanated, fibroblasts were labeled with $^{35}\text{SO}_4$ in the presence or absence of PDGF. CD44 was affinity purified from cell lysates on anti-CD44/protein G/agarose beads and differentially digested with chondroitinase enzymes. In the presence of PDGF both CS and DS glycanation adducts to CD44 were found.

Switching of GAG type in response to a single stimulus such as a growth factor is infrequently encountered, and suggests that the expression or activity of the 5' epimerase, which converts the glucuronate to iduronate (and thereby converts CS to DS), is regulated by PDGF. Previous data suggest that PDGF promotes GAG chain elongation and/or sulfation onto biglycan core proteins of lung fibroblasts (Liu *et al*, 1998). The reported effects were mediated by phosphatidylinositol-3-kinase, a known transducer of PDGF-induced cell migration (Higaki *et al*, 1996).

Although two previous studies demonstrated that CD44 can mediate cell migration into a fibrin gel, substantial differences exist between the previous findings and the data presented here. Henke *et al* (1996b) showed that rabbit microvascular endothelial cell invasive migration into rabbit fibrin was dependent on a CS-CD44 proteoglycan. These cells expressed both CD44H and CD44v3 isoforms. Svee *et al* (1996) demonstrated that lung fibroblasts, isolated from normal individuals and from patients with adult respiratory distress syndrome, required CD44 for invasion of fibrin clots. Similar to the findings in our study, lung fibroblasts expressed only the CD44H isoform (Svee *et al*, 1996). Also in concordance with our findings, the addition of hyaluronidase to fibrin gels did not abrogate the invasive migration of lung fibroblasts (Svee *et al*, 1996). The human lung fibroblast CD44H was not glycanated with CS/DS, however, and the addition of β -xyloside failed to inhibit lung fibroblast invasive migration into fibrin gels (Svee *et al*, 1996), in striking contrast to our findings. As nonglycanated CD44H can bind HA (Lesley *et al*, 1997; Naot *et al*, 1997), but not FN (Jalkanen and Jalkanen, 1992), fibrinogen (Henke *et al*, 1996b), or collagen (Faassen *et al*, 1992), it is not clear what substance in the fibrin gel was ligated by the human lung fibroblast CD44H.

In our previous studies human adult dermal fibroblasts required exogenous FN (Greiling and Clark, 1997). Presumably the galactosaminoglycan adducts on CD44 are necessary for interaction with the FN in the fibrin gels. Jalkanen and Jalkanen (1992) demonstrated that CS glycanation was required for lymphocyte CD44 binding to FN. Furthermore, Barkalow and Schwarzbauer (1991; 1994) identified an arginine pair within heparin-binding site II (Hep II) of FN that is critical for HS and CS binding. This same arginine pair is required for fibroblast migration on FN in our studies (Clark *et al*, 2003). Henke *et al* (1996b) demon-

strated that CS-CD44H binds fibrinogen but did not further analyze the binding site.

Human dermal fibroblast integrins are necessary and sufficient for direct adhesion to either FN (Gailit *et al*, 1993; Gailit and Clark, 1996) or fibrinogen (Gailit *et al*, 1997). Furthermore we have shown that human dermal fibroblast integrins are necessary for invasive migration into an FN/fibrin gel (Greiling and Clark, 1997). From this study, however, it is clear that these cells have additional requirements for invasive migration through an FN/fibrin gel. Previous investigators have shown that, whereas FN binding to integrin receptors is enough to support cell adhesion, FN interaction with integrins is not sufficient for cell spreading and focal contact formation (Woods and Couchman, 1998). For these more complicated processes additional receptors, such as proteoglycans, seem to be necessary. Melanoma cells required both CS proteoglycans and integrins for spreading and focal contact formation (Iida *et al*, 1995). Furthermore, melanoma cell migration on collagen required CS-CD44, as well as the collagen receptor $\alpha 2\beta 1$ integrin, whereas cell adhesion to collagen occurred independently of CS-CD44 (Faassen *et al*, 1992; Knutson *et al*, 1996). In contrast, the predominately HS proteoglycan, syndecan-4, enhanced integrin-mediated focal adhesion formation and transmembrane signaling in fibroblasts (Woods and Couchman, 1998).

Five years ago we reported that fibroblast transmigration from a collagen matrix into a fibrin gel requires FN and depends on both $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins (Greiling and Clark, 1997). In a subsequent study, we demonstrated that human adult dermal fibroblast migration on FN requires three functional domains and $\alpha 4\beta 1$ integrin, as well as $\alpha 5\beta 1$ and $\alpha v\beta 3$ (Clark *et al*, 2003). Here we demonstrate that fibroblasts need proteoglycan receptors, such as DS-CD44H, in addition to integrins, to invade an FN/fibrin gel. Furthermore, post-translational modification of the CD44 with DS must be induced by PDGF, a growth factor found in wounds, for migration to proceed. These data taken together suggest that fibroblast migration is tightly regulated, probably to prevent mesenchymal cell wandering in the absence of wounding. Epithelial, endothelial, nerve, and smooth muscle cells have basement membrane architectural boundaries to prevent cell dispersion. It appears that "naked" mesenchymal cells, such as fibroblasts, have restraints to migration imposed by stringent receptor and ECM requirements.

Materials and Methods

Recombinant PDGF-BB was a kind gift from Charles Hart at ZymoGenetics (Seattle, WA) and its use in our experiment was given institutional approval. FN and thrombin were generously provided by Gerard Marx at New York Blood Center (New York, NY). Bovine serum albumin (BSA, fatty acid free, fraction V) was obtained from Miles (Kankakee, IL). Tissue culture plastic flasks were from Costar (Cambridge, MA). Hanks' balanced salt solution (HBSS), human G-globulin, p-nitrophenyl- α -D-pyranoside (α -xyloside), p-nitrophenyl- β -D-pyranoside (β -xyloside), and collagenase (type I, from *Clostridium histolyticum*) were purchased from Sigma Chemicals (St Louis, MO). Heparinase I, heparinase III (heparitinase I), chondroitinase ACII, chondroitinase B, chondroitinase ABC, hyaluronidase (hyaluronate lyase from *Streptomyces hyalurolyticus*), and

ovomucoid were purchased from Seikagaku America (Ijamsville, MD). Fluorescein isothiocyanate (FITC) microbead calibration standards were supplied by Flow Cytometry Standards (San Juan, PR).

Antibodies Anti-CD44 antibodies A1G3 and A3D8 were a kind gift from Barton Haynes, Duke University Medical Center, Durham, NC (Liao *et al*, 1993; Patel *et al*, 1995; Rivadeneira *et al*, 1995). The antihuman CD44 antibodies BRIC 214 and 235 (Antsee *et al*, 1991) and Hermes-3 (Jalkanen *et al*, 1987) were obtained from the Fifth International Workshop on Leukocyte Differentiation Antigens and came from the Laboratories of Dr D. J. Antsee, International Blood Group Reference Laboratory, Bristol, UK, and Dr S. Jalkanen, Turku University, Finland, respectively. BU52, another anti-CD44 antibody, was purchased from The Binding Site (San Diego, CA). Monoclonal antibodies against human CD44H, CD44v3, CD44v4/5, and CD44v6 were obtained from R&D Systems (Minneapolis, MN). FITC conjugates of goat antimouse IgG was purchased from Caltag Laboratories (South San Francisco, CA).

Fibrinogen Human fibrinogen containing approximately a 1:10 molar ratio of FN (Greiling and Clark, 1997) was obtained from Calbiochem (San Diego, CA). To prevent fibrinolysis, plasminogen was removed from the fibrinogen by passage through a lysine-Sepharose 4B column (Pharmacia, Piscataway, NJ) (Deutsch and Mertz, 1970). The clottability before and after treatment with lysine-Sepharose 4B was over 90%.

Cells Primary cultures of human adult dermal fibroblasts (a kind gift from Marcia Simon, Living Skin Bank, SUNY at Stony Brook) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Gaithersburg, MD), containing 42 mM sodium bicarbonate and supplemented with 100 U per mL penicillin, 100 µg per mL streptomycin, and 10% fetal bovine serum (HyClone, Logan, Utah), at 37°C and 5% CO₂/95% air in a humidified atmosphere. Cells were used between passages 4 and 12.

Normal human keratinocytes were obtained from neonatal foreskins and grown for two passages with lethally irradiated 3T3-J2 cells (Rheinwald and Green, 1975) using a modified medium (Randolph and Simon, 1993). Keratinocyte cultures were characterized and determined to be greater than 99% pure on the basis of characteristic polygonal morphology and immunofluorescence detection of keratin-positive cells. For use cells were grown in serum-free, low-calcium (0.15 mM) medium KGM from Clonetics (San Diego, CA). Experiments were done using fourth passage keratinocytes.

Preparation of floating, contracted collagen gels Fibroblast cultures at 80% confluence were harvested by treatment with 0.05% trypsin/0.01% ethylenediamine tetraacetic acid (EDTA). Trypsin was inactivated by addition of soybean trypsin inhibitor in phosphate-buffered saline (PBS) containing 0.2% BSA. The cells were washed twice with DMEM + 2% BSA and resuspended at a concentration of 1×10^6 cells per mL. The fibroblasts were mixed with neutralized collagen (Vitrogen 100, Celtrix Laboratories, Santa Clara, CA), 2% BSA, 30 ng per mL PDGF-BB, 30 µg per mL FN, and concentrated DMEM so that the final concentration of DMEM was 1×10^6 µL of the cell mixture were added to each well of a 24-well tissue culture plate, which was precoated with 2% BSA, and the collagen was allowed to polymerize at 37°C and 5% CO₂. Final concentration of collagen was 1.8 mg per mL and each gel contained 6×10^4 cells. After 2 h incubation, the gels were gently detached from the plastic surface to allow contraction. 30 ng per mL PDGF-BB in 0.5 mL DMEM + 2% BSA was added, and gels were incubated overnight at 37°C in 5% CO₂/95% air in a humidified atmosphere.

Preparation of dried fibrin fibrils Fibrinogen was mixed with concentrated DMEM and 0.5 U per mL thrombin so that the final concentration of fibrinogen was 300 µg per mL and of 1×10^6 DMEM. Aliquots of 450 µL were added to each well of a 24-well plate, and

after a 2 h incubation at 37°C in 5% CO₂, polymerized gels were dried overnight at 25°C under sterile conditions.

Preparation of three-dimensional transmigration model Dried fibrin fibril-coated dishes were washed once with PBS and contracted collagen gels were placed on the surface. Fibrinogen, at a final concentration of 300 µg per mL, was mixed with DMEM and 1.0 U per mL thrombin. When needed, other supplements such as growth factors were added to the mixture. The collagen gels were surrounded with the fibrinogen solution so that the fibrin gel was level with the collagen gel (Greiling and Clark, 1997).

Evaluation The number of migrated cells was evaluated under a Nikon inverted phase microscope by counting identifiable cell nuclei located outside the contracted collagen gel within the fibrin gel. Each condition was run in triplicate and experiments were repeated at least three times.

Addition of substances to transmigration assay Substances were generally added directly into the fibrinogen solution before addition of thrombin. In experiments with enzyme addition or if a pretreatment with a substance was necessary, the contracted collagen gels were exposed to the enzyme or the substance in the appropriate concentration and for the appropriate period of time before attaching them to the dried matrix. Then, the pretreated collagen gels were attached to the fibrin fibrils and, additionally, the enzyme or substance was added at the same concentration to the fibrin gel. The concentrations and pretreatment times for the substances are as follows: α- and β-xyloside, 1 mM, 24 h; heparinase I and III, 0.1 U per mL, 30 min; chondroitinases AC, B, and ABC, 0.1 U per mL, 30 min.

HA staining In some cell migration assays fibrin gels were treated with 10 U per mL hyaluronidase to clear any HA from the gel that might have been deposited during fibroblast migration. To determine whether any HA was present in gels not treated with hyaluronidase and, if present, whether hyaluronidase had effectively cleared the gels of HA, fibrin gels were stained for HA using a biotin-labeled HA binding fragment (Calbiochem) (Hendin *et al*, 1990). In preparation for this staining, gels were rinsed with PBS three times followed by fixation with 3% paraformaldehyde at 25°C for 25 min and another three washes with PBS. Gels were blocked with 2% BSA in PBS for 2 h and then cells were stained with 20 µg per mL propidium iodide for 2 h at room temperature. After washing with PBS, gels were incubated with biotin-labeled HA binding fragment at 4°C for 48 h. After another wash with PBS, the gels were incubated with Oregon Green 488 conjugated streptavidin at 4°C for 30 min. Unbound streptavidin conjugate was washed away with PBS. Stained specimens were observed and images were captured using a Leica SP2 confocal microscope equipped with a halogen light source and a CCD camera.

DNA fragmentation analysis To identify DNA fragments after exposure to anti-CD44 antibodies in fibrin gel, two protocols were used, those of Henke *et al* (1996a) and Sugano *et al* (1999). Even in the presence of H₂O₂ the primary dermal fibroblasts that we used failed to show any DNA fragmentation with the Henke method, whereas some fragmentation was noted with the Sugano method. Thus, for the data in this report we used the Sugano method. Briefly, fibroblasts in fibrin gels (300 µg per mL) were incubated with 30 ng per mL PDGF with or without 10 µg per mL anti-CD44 antibody or 5 µM H₂O₂ for 24 h at 37°C. Gels were digested with trypsin/EDTA at 37°C, followed by washing to remove fibrin. Cells were then treated with 30 µL of lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 2% Triton-X 100, pH 8.0) for 10 min at 4°C. After centrifugation at 469 g for 20 min, the supernatant was collected and treated for 1 h with RNase A (0.4 µg per mol) at 37°C followed by digestion with proteinase K (0.4 µg per mL). The DNA was separated by electrophoresis on 1.5% agarose gel containing ethidium bromide, visualized, and photographed with a Chemil-mager 4400 system.

Quantitative flow cytometry Fibroblasts and keratinocytes were cultured to 70%–80% confluence. For studies done in monolayer cultures, medium was removed at the beginning of treatment and cells were washed three times with PBS. Fibroblasts were then exposed either to DMEM + 2% BSA or to DMEM + 2% BSA + 30 ng per mL PDGF, whereas keratinocytes were cultured in KGM + 2% BSA \pm 30 ng per mL PDGF. After 48 h the cells were assayed for CD44 isoform expression by quantitative flow cytometry essentially as previously described (Gailit and Clark, 1996).

In addition, fibroblasts were embedded in collagen gels + 2% BSA and 30 ng per mL PDGF as was done for the transmigration assay. Then, the contracted collagen gels were incubated with DMEM + 2% BSA or with DMEM + 2% BSA and 30 ng per mL PDGF. After 48 h at 37°C and 5% CO₂/95% air in a humidified atmosphere the gels were digested by incubation with 1 mg per mL collagenase for 1 h in a shaking waterbath. After centrifugation and resuspension in PBS the cells were assayed for CD44 isoform expression.

In both situations resuspended cells were mixed 4:1 (final cell concentration 1×10^6 cells per mL) with a blocking solution containing 10 mg per mL human γ -globulin and 0.2% sodium azide in HBSS. After incubation for 10 min on ice, 4×10^5 cells were transferred to tubes and washed twice with FC medium. The cells were then resuspended in 100 μ L of primary antibody diluted in FC medium (1:1000 each) and incubated for 60 min on ice. Then, cells were washed twice with 2 mL cold FC medium (hanks with 0.2% BSA and 0.2% oxide). They were resuspended in 50 μ L of secondary antibody diluted in FC medium (FITC conjugates of goat antimouse, diluted 1:50), mixed gently, and incubated for 30 min on ice. The cells were washed once with 2 mL cold FC medium and then fixed for 20 min on ice with 0.5 mL of PBS containing 1% paraformaldehyde. After fixation, 2 mL cold PBS containing 0.2% sodium azide was added to each tube. The tubes were centrifuged to pellet the cells and the cells were resuspended in 0.5 mL cold PBS containing 0.2% sodium azide.

Cell surface expression of CD44 isoforms was analyzed by quantitative flow cytometry performed with a FACStar Plus cell sorter (Becton Dickinson Immunocytometry Systems, San Jose, CA) running LYSYS II software. Samples were gated by forward and side scatter to exclude cell aggregates and cell debris. FITC microbead standards allow for the conversion of relative fluorescence intensity into an absolute value, the number of molecules of equivalent soluble fluorochrome (MESF). The results reported here are based upon the calculated MESF value for each experimental sample minus the MESF value for the appropriate antibody control.

Western blot of proteoglycans This procedure was done with slight modification as described earlier (Couchman *et al*, 1996). Briefly, fibroblasts were washed in serum-free medium and then incubated for 45 min at 37°C in HBSS lacking phosphate but buffered with 30 mM sodium acetate at pH 7.4. Some cultures were treated with chondroitinase ABC or ACII in this buffer (0.2 U per mL), whereas others were treated with 0.1 μ L chondroitinase B. After this treatment monolayers were lysed directly in 100 μ L of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 20 mM dithiothreitol, before heating at 100°C for 3 min. Samples were run on 4%–15% SDS-PAGE and transblotted to nitrocellulose as previously described. The blots were blocked and processed as described (Couchman *et al*, 1996). Blots were probed with an antibody against CD44H (R&D Systems, 2 μ g per mL), R36, polyclonal antibodies raised against chondroitinase ABC digested aggrecan from bovine nasal cartilage (Couchman *et al*, 1996), or with a mixture of 5 μ g per mL each of anti- Δ Di-OS, Δ Di-4S, and Δ Di-6S monoclonal antibodies (Seihaguku America) (Couchman *et al*, 1984). R36 and the mixture of monoclonal antibodies recognize chondroitin/dermatan “stubs” generated by chondroitinase treatment. Secondary antibodies were goat antimouse IgG or goat antirabbit IgG coupled

to horseradish peroxidase (Bio-Rad, Hercules, CA dilutions 1:3000). The presence of peroxidase was determined by chemiluminescence (Amersham).

Sulfated GAG synthesis Fibroblasts were cultured with ³⁵SO₄ (50 mCi per mL) for 48 h at 37°C in DMEM containing 10% fetal bovine serum in the absence or presence of PDGF (100 ng per mL). After washing with cold PBS, the cells were dissolved in immunoprecipitation buffer (50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 0.75 mM CaCl₂, 1% Triton X-100, and protease inhibitors). Lysates were spun down and supernatants were incubated with protein-G-conjugated agarose beads for 90 min at 4°C to remove non-specific binding. After centrifugation, supernatants were incubated with anti-CD44H antibody, which had been linked to protein G/agarose beads, for 90 min at 4°C. After washing thoroughly, the beads were digested with chondroitinase ABC, AC, or B (0.2 U per mL) for 24 h at 37°C, followed by digestion with NaOH/NaBH₄ for 24 h at 37°C. Reaction mixtures were adjusted to pH 7.4 with HCl and passed through a G-50 Sephadex column, eluted with 0.5 M NaCl, 20 mM Tris–HCl, pH 7.4, and 0.1% Triton X-100. Fractions were collected and counted with a liquid scintillation counter.

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